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TITLE: Regulation of Estrogen Receptor mRNA Stability in Human Breast Cancer Cells

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The growth and metastases of approximately 40% of human breast cancers is dependent on 17β-estradiol-estrogen receptor (E_2-ER) complex. ER levels are controlled, in part, through an autoregulatory loop in which E_2-ER complex (and mitogens) down-regulate ER mRNA levels. We have created tetracycline (TET)-regulated expression plasmids which transcribe either the full length 6.4 kb human ER mRNA or truncated 'mini-ER' mRNAs, identified improved methods for delivering the plasmids encoding these mRNAs to cells in transient transfections, and developed a novel quantitative PCR procedure, which allows quantitative PCR of RNA from cells transiently transfected with DNA plasmids encoding the RNA. Utilizing both the TET system and quantitative RT-PCR, we demonstrated that TPA was unable to post-transcriptionally destabilize ER mRNA in MDA-MB-231 breast cancer cells transiently transfected with the TET-regulated full length human ER expression vector. We have shown that the TET-regulated full length ER expression plasmid produces functional ER protein in cell culture which is estrogen-responsive and activates the 2ERE-PS2-CAT expression vector in MDA-MB-231 cells. We have developed new techniques for measuring mRNA degradation and are currently determining the extent to which destabilization contributes to ER mRNA down-regulation in human breast cancer cells.

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FOREWORD

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J P. Pippo

PI - Signature Date

Annual Summary (Final Report) for Grant Number DAMD 17-96-1-6177: September 1, 1996 - September 1, 1998

Due Date: October 1, 1998

Description of Training:

1) Investigated the regulation of estrogen receptor (ER) mRNA stability in ER-positive (MCF-7) and ER-negative (MDA-MB-231) human breast cancer cell lines using both the global transcriptional inhibitor, actinomycin D, and the more specific, tightly controlled tetracycline (TET) regulated expression vector system, respectively.

2) Gained significant experience in measuring and quantitating ER mRNA levels using conventional (Northern blotting) and more recent (quantitative PCR) methods for mRNA detection.

3) Beyond standard cDNA cloning methods, expanded knowledge of cloning techniques including "long" RT-PCR (useful in generating a full length ER PCR product > 6 kb) and "quick change" site-directed mutagenesis (exploited for primer specificity in subsequent quantitative PCR experiments).

4) Utilized a number of different and unique methods for transiently transfecting human breast cancer cell lines (including: calcium-phosphate precipitation, electroporation, liposome, and transferrin-lipofectin delivery systems).

5) Attended the Department of Defense Breast Cancer Research Program Meeting in Washington D.C. (October 31-November 4, 1997), the 80th Annual Meeting of the Endocrine Society in New Orleans (June 24-27, 1998), and all relevant on campus seminars at the University of Illinois, Urbana-Champaign relating to ER and breast cancer research, which considerably increased my understanding and knowledge of the many basic science and clinical issues related to breast cancer (including: etiology, prevention, detection, and treatment possibilities).

Accomplishments to Date:

1) Conducted experiments using actinomycin D and Northern blotting to reproduce the observations made previously by others that estrogen and 12-O-tetradecanoylphorbol-13-acetate (TPA) down regulate ER mRNA in MCF-7 breast cancer cells, in part, by post-transcriptional destabilization.

2) Created "mini ER" clones and a functional full length ER clone (6.45 kb) containing the entire 4 kb 3'-untranslated region which can be: a) expressed *in vivo* using the TET expression vector system, b) bind to estrogen response elements (EREs) located within a 2ERE-PS2-CAT reporter and c) increase CAT activity in the presence of estrogen.

3) Established an experimental design to measure mRNA stability which combines the effectiveness of the TET system with the sensitivity of quantitative RT-PCR technology modified for use with RNA samples from transiently transfected cells.

4) Presented the following preliminary results at the 80th Annual Meeting of the Endocrine Society in New Orleans (June 24-27, 1998):

   a) Utilizing both the TET system and quantitative RT-PCR, we demonstrated that TPA was unable to post-transcriptionally destabilize ER mRNA in MDA-MB-231 breast cancer cells transiently transfected with the TET-regulated full length human ER expression vector. In the presence or absence of TPA, the half-life of ER mRNA was calculated to be approximately 4.5 hours (consistent with the t1/2 of ER mRNA calculated when performing stability experiments using actinomycin D).

   b) The "TET-off" system when employed in transient transfection remains under tight control. In the constant presence of tetracycline (2 μg/ml), transcriptional activity of TET-LUC reporter gene was kept below 2% of maximal luciferase activity when compared to luciferase expression in the absence of tetracycline.

   c) Also using luciferase assays, we determined that TPA had no effect on the TET-promoter (unlike the effect on a CMV-promoter) to increase transcriptional activity. Thus, we ruled out the possibility that TPA was masking its own destabilizing effects by activation of the TET-promoter driving ER mRNA expression.

Discussion (as related to scientific content and address to the Statement of Work (SOW)):

As a result of this research, we anticipate publishing both the novel approach to measuring mRNA levels using the TET system coupled to quantitative PCR as well as any future studies which help further characterize the ER mRNA destabilization effect of estrogen or TPA. The tasks proposed within the SOW are still in progress due to two critical factors. The first, which was mentioned in last year's annual report, was the necessity to establish a method in our laboratory for quantitating mRNA levels which would be both accurate and sensitive enough to detect mRNA produced from transiently transfected expression vectors. The second factor was our inability to observe a post-transcriptional destabilizing effect thus far under the conditions mentioned above (Accomplishments to Date: 4a).

Continued efforts to consistently detect significant levels of TET-expressed ER mRNA in MCF-7 cells were unsuccessful due to a decreased transfection efficiency (when compared with MDA-MB-231 transfection efficiency). However, future reliable detection of ER mRNA generated from transfected TET constructs in MCF-7 cells will quickly allow us to reproduce the ER mRNA destabilization effect and then subsequently proceed with the characterization of mRNA sequences and binding proteins necessary for the effect as stated in the SOW.

On a personal note, I would like to thank the Army Breast Cancer Research Program for their postdoctoral research award. This grant allowed me to greatly expand my knowledge and expertise in molecular and cellular biology as it relates to the fields of mRNA stability and breast cancer research. I received an enthusiastic response at my well attended poster presentation of our research findings at the ENDO meeting in New Orleans in June of this year. Furthermore, I remain confident and hopeful that the preliminary work completed during the past two years will both, be published and also become the basis for further studies and publications in the field of breast cancer research. Successful identification of ER mRNA stabilizing and destabilizing proteins can potentially have a highly significant impact on the regulation of ER status, thus creating new opportunities both in drug design and treatment modalities designed for breast cancer patients.